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Identification of the Herpes Simplex Virus DNA Sequences Present in Six Herpes Simplex Virus Thymidine Kinase-Transformed Mouse Cell Lines

JEFFREY M. LEIDEN,[1] NIZA FRENKEL,[1]* AND FRED RAPP[2]

Department of Biology, The University of Chicago, Chicago, Illinois 60637,[1] and Department of Microbiology and Specialized Cancer Research Center, The Milton S. Hershey Medical Center, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033[2]

We have used a novel filter hybridization approach to detect and map the herpes simplex virus (HSV) DNA sequences which are present in four HSV thymidine kinase (HSVtk$^+$)-transformed cell lines which were derived by exposure of thymidine kinase negative (tk$^-$) mouse cells to UV light-irradiated HSV type 2 (HSV-2). In addition, we have mapped the HSV-1 DNA sequences which are present in two HSV-1tk$^+$-transformed cell lines produced by transfection of tk$^-$ mouse cells with sheared HSV-1 DNA. The results of these studies can be summarized as follows. (i) The only HSV DNA sequences which were common to all HSVtk$^+$-transformed cells were those located between map coordinates 0.28 and 0.32. Thus, this region contains all of the viral DNA sequences which are necessary for the expression of HSV-mediated tk transformation. (ii) Many of the cell lines also contained variable amounts of non-tk gene viral DNA sequences located between map coordinates 0.11 to 0.57 and 0.82 to 1.00, suggesting that incorporation of the viral DNA sequences located between these map coordinates is a relatively random event. (iii) The viral DNA sequences located between map coordinates 0 to 0.11 and 0.57 to 0.82 were uniformly absent from all of the HSVtk$^+$ cell lines tested, suggesting that there is a strong negative selective pressure against incorporation of these viral DNA sequences.

During their lytic cycle, herpes simplex viruses have been shown to program the synthesis of a virus-specific thymidine kinase (tk) enzyme (6, 15). This tk has been shown to be distinct from both the nuclear and mitochondrial mouse and hamster cell enzymes with respect to a number of biochemical and immunological properties, including thermal stability, electrophoretic mobility, and antigenic specificity (16, 26). The enzyme has been shown to belong to the β class (11) of viral proteins, in that its synthesis requires the presence of functional early (α) viral polypeptides and is turned off late in infection by late (γ) viral polypeptides (7, 18; R. Honess and B. Roizman, unpublished observations).

Munyon et al. (27) were the first to show that a small proportion of tk-negative (tk$^-$) mouse cells could be stably transformed to the tk-positive (tk$^+$) phenotype after exposure of the tk$^-$ cells to UV light-irradiated herpes simplex virus type 1 (HSV-1). The tk enzyme made in such biochemically transformed cells has been shown to possess the biochemical and immunological characteristics of the tk produced during lytic HSV infections (4, 17, 26, 34).

More recently, transformation of tk$^-$ mouse cells to the tk$^+$ phenotype has been accomplished by transfecting the tk$^-$ cells with either sheared HSV DNA, or isolated restriction enzyme fragments of HSV DNA (1, 21, 23, 37). By using these approaches, Wigler et al. (37) have shown that a 3.4-kilobase fragment of HSV-1 (F) DNA is sufficient to stably transform tk$^-$ cells to the HSV tk-positive (HSVtk$^+$) phenotype. Furthermore, Maitland and McDougall (21) have reported that the purified restriction enzyme fragments of HSV-2 (333) DNA which contain the viral DNA sequences located between map coordinates 0.53 and 0.65 are sufficient to transform tk$^-$ mouse cells to the HSVtk$^+$ phenotype. This map location for the HSV-2 tk-transforming gene was surprising in light of recent studies of HSV-1 × HSV-2 recombinants which have revealed that most, if not all, of the genes of these two viruses are colinear (24, 25, 29), and which have mapped the HSV-1 tk gene between coordinates 0.27 and 0.35 (25).

The studies described in this paper were designed to both unambiguously map the HSV-1 and HSV-2 tk genes and to study the patterns of incorporation of non-tk gene viral DNA sequences in HSVtk$^+$ transformants produced by infection of tk$^-$ mouse cells with UV light-irradiated HSV or by transfection of these cells with

sheared viral DNA. Toward these ends we have developed a novel hybridization mapping approach which has allowed the detection and identification of the HSV DNA sequences which are present in a number of HSVtk$^+$ cell lines. Our results have revealed that (i) the only region of the HSV genome which is present in all HSVtk$^+$-transformed cells is located between map coordinates 0.28 and 0.32, and (ii) a wide variety of non-tk gene viral DNA sequences can be stably incorporated into the HSVtk$^+$ cells. However, specific regions of the HSV genome appear to be uniformly absent from such HSVtk$^+$ transformants.

MATERIALS AND METHODS

Virus. HSV-2 strains 333 and 324 (both isolated from penile lesions) and Silow (isolated from a vaginal lesion) were obtained from W. Rawls (McMaster University, Hamilton, Ontario, Canada). HSV-1 (1023) is a recombinant between HSV-1 (HFEM) and HSV-1 (MP) (32). HSV-2 strain G, HSV-1 strains F and MP, and HSV-1 (1023) were obtained from B. Roizman (University of Chicago, Chicago, Ill.). HSV-1 (Justin) was obtained from A. Sabin.

HSVtk$^+$-transformed mouse cells. HSVtk$^+$-transformed cell lines 33A$^+$, 39A$^+$, 59D$^+$, and Silow were obtained by the biochemical (tk) transformation method of Munyon et al. (27), as modified by Rapp and Turner (30). NclA c110 (tk$^-$) cells, inbred Swiss mouse cells lacking tk activity, were originally obtained from R. Goldberg (National Institutes of Health, Bethesda, Md.). The stocks of HSV-2 to be used for transformation were UV-irradiated for 5 min at 46 ergs/s per mm^2. This UV-irradiated virus was then used to infect the NclA c110 cells in suspension (6×10^5 cells per 100-mm plate) at a multiplicity of 2 to 4 PFU/cell (as calculated before UV irradiation). After 72 h, selective medium containing MTAGG (0.28 μg of methotrexate sodium per ml, 4 μg of thymidine per ml, 13 μg of adenosine per ml, 14 μg of guanosine per ml, and 7.5 μg of glycine per ml) replaced nonselective medium (27). Three weeks after infection, foci of tk$^+$-transformed cells appeared on the infected plates and were picked with a Pasteur pipette. Cell lines were grown and continuously propagated in MTAGG-containing medium. HSVtk$^+$ cell lines 33A$^+$ and 39A$^+$ were transformed by HSV-2 (333), cell line 59D$^+$ was transformed by HSV-2 (324), and cell line Silow was transformed by HSV-2 (Silow). Cells were passaged at least 50 to 100 times (every 4 to 5 days) before being used in the studies described in this paper. Cell lines 8N and 5A were obtained from D. Polacek and B. Roizman. These cell lines were produced after transfection of L tk$^-$ Cl1D cells with sheared HSV-1 (1023) DNA, by using the calcium-phosphate precipitation method of Graham et al. (8). After transfection, the cells were switched to Littlefield's HAT medium (0.01 M hypoxanthine, 4.4 μM methotrexate, and 16 μM thymidine) (20). Surviving colonies were picked by using small glass cylinders and were further propagated in HAT-containing medium. Passage 15 of each of these cell lines was used in the experiments described below.

Assay of transformed-cell tk activity. HSV-2tk$^+$ cells between passages 50 to 100 were assayed for tk activity by using the method of Lin and Munyon (19) as modified by Rapp and Turner (30). Both noninfected and infected (18 h with HSV-2 (333), at 2 PFU/cell) control cultures, composed of normal NclA c110 (tk$^-$) and NIH Swiss mouse (tk$^+$) cells, were also assayed.

To selectively inactivate the viral tk activity, cell extracts were incubated at 40°C for 30 min (28).

Purification of viral DNA. Purification of viral DNA was performed as follows. Vero cells were infected with 5 PFU/cell of HSV in 199-V medium containing 1.5 μCi of [^{3}H]thymidine (Amersham Corp.) per ml. After 20 h at 37°C, the infected cells were scraped into the medium and pelleted. The cell pellet was rinsed twice with cold phosphate-buffered saline and resuspended in lysis buffer (0.1 M NaCl, 0.01 M Tris-hydrochloride (pH 8.0), 0.01 M EDTA; 13 ml/10^9 cells). Sodium dodecyl sulfate (SDS) and pronase (Sigma Chemical Co.), pretreated according to Hotta and Bassel (12), were added to final concentrations of 0.6% and 1 mg/ml, respectively, and the mixture was incubated at 37°C for 4 to 6 h. The lysis mixture was then diluted to a final volume of 150 ml with 0.01 M Tris-hydrochloride (pH 8.0)–0.001 M EDTA (TE) and added to 195 g of solid CsCl (Eastern Chemical). The refractive index of the resulting solution was adjusted to 1.4005, and the mixture was centrifuged at 40,000 rpm for 20 h at 20°C in a Beckman VTi50 rotor in an L-5 centrifuge. Fractions containing viral DNA were combined. The refractive index of this pooled peak material was readjusted to 1.4005 with a CsCl solution, and the resulting mixture was recentrifuged to equilibrium as described above. The viral peak material from these second gradients was pooled and dialyzed against TE buffer.

Mapping approach. The mapping approach which has been used to identify the viral DNA sequences present in the HSVtk$^+$ cell lines is schematically represented in Fig. 1. Basically, this approach involves three sequential steps: (i) an initial density gradient centrifugation step of the transformed-cell DNA, to enrich for transformed cell-associated viral DNA sequences, (ii) in vitro labeling of the gradient-selected DNA followed by a preselection hybridization to HSV DNA immobilized on nitrocellulose filters to further enrich for the cell-associated viral DNA sequences, and (iii) hybridization of the preselected transformed-cell DNA to blots containing restriction enzyme fragments of HSV DNA.

(i) Extraction and CsCl density gradient centrifugation of transformed-cell DNA. DNA from either 1.25×10^8 or 2.5×10^8 cells was labeled in vivo by growing the cells for 2 days in the presence of 0.5 μCi of [^{3}H]thymidine (Amersham Corp.) per ml. The cells were harvested by rinsing the monolayers three times with ice-cold phosphate-buffered saline, followed by lysis in 25 ml of a mixture containing 0.01 M Tris-hydrochloride (pH 8.0), 0.01 M EDTA, 0.01 M NaCl, 0.5% SDS, and 1 mg of Proteinase K (Merck & Co., Inc.) per ml. The lysis mixture was incubated overnight at 37°C and sheared on ice five times (30 s/time) at 20,000 rpm in a VirTis 45 homogenizer. The sheared DNA was extracted with 1 volume of phenol and 1 volume of chloroform and was dialyzed at room

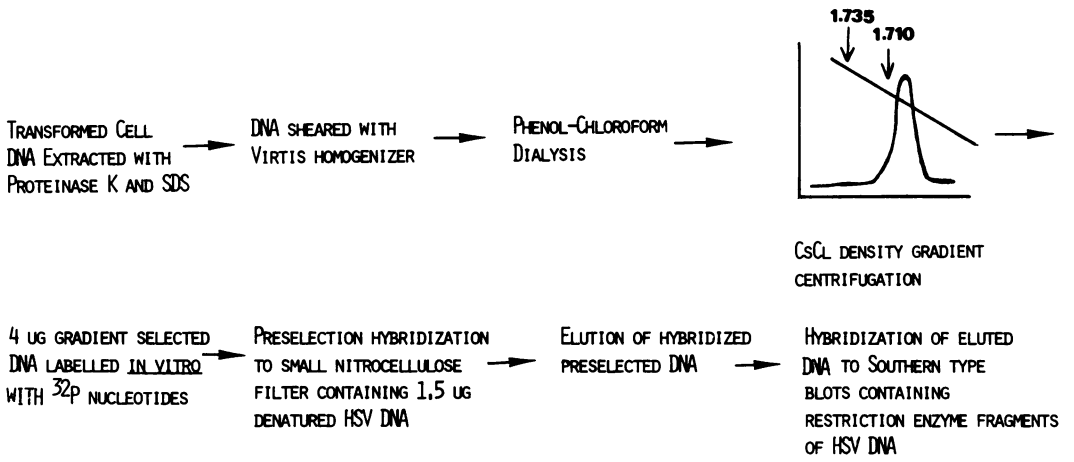

FIG. 1. *Diagrammatic representation of the hybridization approach. The individual steps of this approach are described in detail in the text.*

temperature for approximately 12 h against TE buffer. The DNA mixture was then resheared five times as above, adjusted to a total volume of 25 ml, and added to 32.5 g of solid CsCl (Eastern Chemical) to yield a final refractive index of 1.4005. Equilibrium density centrifugation was done in a VTi50 Beckman rotor at 40,000 rpm at 20°C for 20 h. Sixty 600-μl fractions were collected per gradient. The fractions between densities 1.710 and 1.735 g/cm^3 (as determined from refractive indices) were pooled and dialyzed against several changes of TE buffer at 4°C for 3 days. The DNA was then precipitated with ethanol. Figure 2 shows the results of a reconstruction experiment in which a mixture of purified ^{32}P-labeled HSV-2 DNA and ^{3}H-labeled tk$^-$ cell DNA was sheared and subjected to equilibrium density centrifugation as described above. As shown in Fig. 2, the sheared viral and cell DNAs sedimented in discrete bands. The fractions between densities 1.710 and 1.735 g/cm^3 contained 75% of the ^{32}P-viral DNA label and only 2% of the ^{3}H-labeled cellular DNA, representing a 40-fold enrichment.

(ii) In vitro labeling of DNA. Viral DNA and CsCl gradient-selected transformed-cell DNAs were labeled in vitro with [α-^{32}P]dGTP and [α-^{32}P]dCTP (350 Ci/mmol; Amersham Corp.) with *Escherichia coli* DNA polymerase I, essentially as described by Maniatis et al. (22). The specific activities of DNA labeled by this procedure ranged between 7×10^7 and 1.6×10^8 cpm/μg.

(iii) Preparation of nitrocellulose filters containing HSV DNA. The small nitrocellulose filters used in the preselection hybridization step were prepared as follows. A 10- to 15-μg amount of HSV-1 or HSV-2 DNA was suspended in 0.9 ml of 1× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate) and sheared five times through a 26-gauge needle. A 0.1-ml amount of 3 N NaOH was added, and the mixture was incubated for 10 min at room temperature. This mixture was then added to 9 ml of 28× SSC and filtered two times through a HAWP 02500 nitrocellulose membrane filter (Millipore Corp.), which had been prewashed with 28× SSC. After filtration, the filter was washed with 10 ml of 28× SSC, dried overnight at room temperature, and baked at 80°C for 5 h. Eight replicate 2-mm-diameter filters were punched out of each 25-mm-diameter filter, with a leather punch. It was found that filtration in alkali as originally described by Baker (2) along with the use of very high salt (28× SSC) resulted in the best retention of HSV DNA.

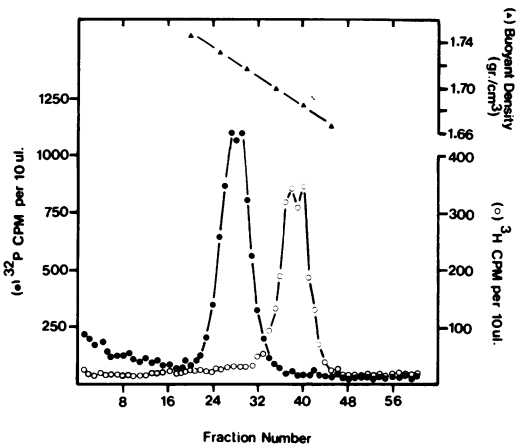

FIG. 2. *CsCl equilibrium density centrifugation of a mixture of sheared tk$^-$ cell DNA and HSV-1 DNA. A mixture of ^{32}P-labeled HSV-1 DNA and ^{3}H-labeled Cl1D tk$^-$ cellular DNA was sheared to an average molecular weight of 5×10^6 and sedimented to equilibrium in a CsCl density gradient as described in the text.*

Nitrocellulose "blots" containing various restriction enzyme fragments of HSV DNA were prepared as follows. HSV DNA was digested with restriction endonucleases (New England Biolabs), and subjected to

electrophoresis at 50 V for 20 h in a horizontal agarose gel (10 by 30 cm; 0.4%), as described by Hayward (10). A 10- to 15-μg amount of HSV DNA was loaded onto 4-cm-wide wells. DNA was transferred from the gel onto a sheet of BA 85 nitrocellulose filter (Schleicher & Schuell Co.), essentially as described by Southern (33). After blotting, replicate blots were prepared by cutting the nitrocellulose filter into 3-mm vertical strips which were allowed to dry overnight at room temperature and then baked at 80°C for 5 h.

(iv) Preselection hybridizations. Nitrocellulose filters (2-mm diameter), prepared as described above and containing 1.5 μg of immobilized denatured HSV DNA, were preincubated for 6 h at 65°C in a solution containing 6× SSC and 0.02% bovine serum albumin (BSA), 0.02% polyvinylpyrrolidone, and 0.02% Ficoll (5).

In vitro-labeled transformed-cell DNA (4 μg) was precipitated with ethanol in a 1.5-ml microfuge tube and resuspended by heating at 65°C for 30 min in 20 μl of hybridization solution containing 6× SSC, 0.01 M EDTA, 30% formamide, and 1 mg of *E. coli* DNA (Miles Laboratories, Inc.) per ml. The resuspended DNA was then sealed in a capillary pipette and denatured for 7 min at 117°C. The resulting denatured DNA was placed into a Beem capsule (Beem Corp.), and 2 μl of 10× Denhardt solution (1× Denhardt solution is 0.02% BSA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone) in water was added. A preincubated nitrocellulose filter (2-mm diameter) containing immobilized HSV DNA was blotted dry and submerged in the hybridization solution at the bottom of the Beem capsule. The filter and hybridization solution were overlaid with mineral oil, and hybridization was carried out at 65°C for 16 h. After hybridization the small nitrocellulose filter was washed three times at room temperature with 2× SSC and twice for 1 h at 65°C in 10 ml of 6× SSC–30% formamide–1× Denhardt solution–0.5% SDS. After these washes, the hybridized DNA was eluted from the filters by incubation of each filter in 100 μl of elution buffer (0.1 M NaOH, 0.01 M EDTA, 0.1% SDS) at 45°C for 2 h, as originally described by Groneberg et al. (9). The solution of eluted DNA was neutralized with 1 N HCl. It should be noted that the efficiency of the preselection hybridization step was greatly improved by minimizing the volume of the hybridization solution. For convenience, we chose a final volume of 20 μl per filter, yielding a 50% hybridization efficiency for purified viral DNA and a background of 0.02 to 0.04% for cellular DNA and, therefore, representing a 1,200- to 2,500-fold enrichment for viral DNA sequences.

(v) Blot hybridizations. Nitrocellulose blots were preincubated for 6 h at 65°C in a 1× Denhardt solution in 6× SSC. These preincubated filter strips were then hybridized in a glass scintillation vial containing the eluted DNA from the preselection hybridization step in 1 ml of a solution containing 6× SSC, 1× Denhardt solution, 30% formamide, 10% dextran sulfate (Pharmacia Fine Chemicals, Inc.), 0.5% SDS, and 200 μg of denatured *E. coli* DNA per ml. Hybridization was carried out at 65°C under mineral oil with vigorous shaking for 20 h. It should be noted that the inclusion of 10% dextran sulfate (36) into the blot hybridization mixtures was found to result in a two- to fourfold increase in the hybridization efficiency. In addition, it was found that the incorporation of excess unlabeled *E. coli* DNA into the hybridization solutions resulted in significantly reduced levels of background, as compared to hybridizations performed in the presence of excess unlabeled salmon sperm DNA. After hybridization, the nitrocellulose strips were washed three times at room temperature with 2× SSC and twice for 2 h with 6× SSC–1× Denhardt solution–30% formamide–0.5% SDS at 65°C. Finally, the nitrocellulose strips were dried and autoradiogrammed on Cronex 2_C^D medical X-ray film (Du Pont Co.) either with (at −70°C), or without (at room temperature) high speed intensifying screens (Du Pont Co.). When applicable, the relative intensities of autoradiographic bands were determined by scanning the autoradiograms with a Transidyne 2955 scanning densitometer equipped with an automatic integrator.

Theoretical treatment of the fractional recovery of integrated viral DNA from the CsCl equilibrium density centrifugation step. Consider a piece of HSV DNA of average viral buoyant density, ρ_v (= 1.729 g/cm^3) (14), and size V, which is integrated into cellular DNA sequences of average buoyant density ρ_c (= 1.690 g/cm^3). After random shearing of the transformed-cell DNA to double-stranded fragments of size L, any integrated sequence of viral DNA will be present in an assortment of pieces of sheared DNA, containing various amounts of viral and cellular sequences. In order for a piece of this sheared transformed cell DNA to be recovered from the CsCl equilibrium density centrifugation step (i.e., to display a buoyant density greater than or equal to ρ = 1.710 g/cm^3), it must contain a minimum fraction of viral DNA sequences. This minimum fraction (f) can be easily calculated from the following equation: $\rho = (f)\cdot\rho_v + (1 - f)\rho_c$ (equation 1). It follows that for a piece of transformed-cell DNA of length L, Lf represents the minimum amount of viral DNA which must be contained in that piece if it is to be recovered from the density gradient preselection step.

Assuming that ρ_v = 1.729 g/cm^3, ρ_c = 1.690 g/cm^3, ρ = 1.710 g/cm^3, and $L = 6 \times 10^6$ (i.e., the average size of the sheared transformed-cell DNA), then from equation 1 $f = 0.5$ and $Lf = 3 \times 10^6$. Thus, any piece of integrated viral DNA of molecular weight V less than 3×10^6 will not be recovered from the CsCl gradient purification step. On the other hand, when the size of an integrated piece of viral DNA is greater than 3×10^6, all of the viral DNA sequences contained in this piece of integrated DNA will be recovered to some extent during the CsCl purification step. The exact fractional recovery of a given viral DNA sequence will depend upon its position X along the stretch of integrated viral DNA. The relationship between the fractional recovery F and the position X, for the viral DNA sequences contained in various pieces of integrated viral DNA of sizes $V > Lf$ is shown in Fig. 3. As seen in that figure, those viral DNA sequences located at both edges of the piece of integrated viral DNA (i.e., at $X = 0$ and $X = V$) will be recovered from the equilibrium density step with an efficiency of $F = 1 - f$ which is independent of both L, the size of the sheared transformed-cell DNA, and V, the size of the integrated viral DNA sequence. As described above

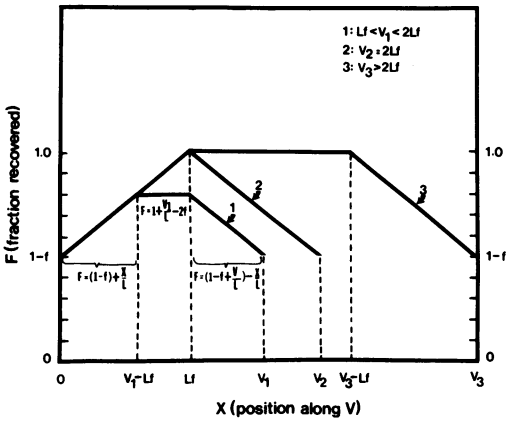

FIG. 3. *Diagrammatic representation of the fractional recovery of integrated viral DNA sequences as a function of the position of the integrated viral DNA sequences. L = average length of sheared transformed cell DNA, X = distance of a given viral DNA sequence from one end of the integrated piece of viral DNA, F = fractional recovery from CsCl equilibrium density gradient of a given viral DNA sequence located at position X, f = minimum fraction of a piece of sheared transformed-cell DNA which must be composed of viral DNA sequences if that piece of transformed cell DNA is to be recovered from the CsCl equilibrium density centrifugation step, and V = the size of an integrated stretch of viral DNA. As described in the text, a given viral DNA sequence will be included in an assortment of fragments of randomly sheared transformed-cell DNA. F was calculated as the fraction of these fragments which contain at least Lf amount of viral DNA sequences. F was calculated for three cases differing with respect to the size V of the integrated viral DNA as depicted in the upper right hand corner of the figure.*

using our experimental conditions $f = 0.5$. Therefore, those viral DNA sequences located at both edges of the piece of integrated viral DNA will be recovered with an efficiency $F = 0.5$. Viral DNA sequences located at internal positions of the integrated stretch of viral DNA ($V > X > 0$) will be recovered with efficiencies which vary linearly between $1 - f$ (= 0.5) and 1.0. In summary, by using our experimental conditions this theoretical treatment predicts that all of the viral DNA sequences present in integrated pieces of viral DNA with molecular weights greater than 3 $\times 10^6$ will be recovered with a minimum efficiency of 50%.

RESULTS

Biochemical characterization of HSVtk$^+$ transformed cell lines. HSV-2tk$^+$ transformants were obtained as described above after exposure of Nc1A c110 (tk$^-$) mouse cells to several strains of UV light-irradiated HSV-2 (30). The resultant tk$^+$ colonies were continuously propagated in MTAGG selective medium. The various cell lines were assayed for tk activity after 50 to 100 passages. These tests (Table 1) have shown that all of the cell lines express a tk activity which displays the characteristic thermal lability of the HSV-2 enzyme (28).

HSV-1tk$^+$ transformants were produced by transfecting L tk$^-$ Cl1D mouse cells with HSV-1 (1023) DNA which was randomly sheared to fragments of molecular weight 20 $\times 10^6$ to 30 $\times 10^6$ (D. Polacek and B. Roizman, unpublished data). After 24 h, the cells were placed in Littlefield's HAT medium (20) to select for tk$^+$ cells. Surviving colonies were picked after 2 to 3 weeks and grown continuously for 15 to 20 passages in HAT-containing medium.

Characterization of the mapping approach. The mapping approach which we developed (Fig. 1) involves three sequential steps. In the first step, transformed-cell DNA is randomly sheared to fragments of molecular weight 5 $\times 10^6$ to 7 $\times 10^6$ and subjected to CsCl equilibrium density centrifugation. Because HSV and cellular DNAs display widely different buoyant densities (1.726 and 1.729 g/cm^3 for HSV-1 and HSV-2 DNAs, respectively, [14] and 1.69 to 1.70 g/cm^3 for cellular DNA), this step results in the partial purification of any viral DNA sequences which are present in the transformed cells. In the second step, the DNA banding at densities greater than or equal to 1.710 g/cm^3 is labeled, in vitro, with α-^{32}P-nucleotides and further enriched for viral DNA sequences by hybridization to a small nitrocellulose filter containing denatured unlabeled HSV DNA. Finally, the hybridized, labeled transformed cell DNA is eluted from the filters and rehybridized to Southern type blots containing unlabeled restriction enzyme fragments of HSV DNA (33). The resulting bands are visualized by autoradiography and are

TABLE 1. *Viral tk activity in biochemically transformed mouse cells*

Cell extract	nmol of [^{3}H]TdR incorporated per mg of protein in 45 min[a]		Residual activity (%)
	No preincubation	Preincubation at 40°C[b]	
Nc1A c110 (tk$^-$)	0.029	0.043	148.3
Nc1A c110 (tk$^-$) + HSV-2 (333)	0.30	0.081	27.0
NIH Swiss mouse (tk$^-$)	0.20	0.18	90.0
NIH Swiss mouse (tk$^-$) + HSV-2 (333)	0.81	0.15	18.5
33A$^+$ cells	0.27	0.039	14.4
39A$^+$ cells	0.23	0.066	28.7
59D$^+$ cells	0.48	0.11	22.9
Silow cells	0.35	0.057	16.3

[a] The assay for thymidine kinase was performed as described in the text. TdR, Thymidine.

[b] Preincubation was performed for 30 min.

identified by comparison to autoradiograms of replicate control blots which were hybridized to purified, in vitro-labeled HSV DNA.

Specificity and sensitivity of the mapping approach. To assess the specificity of the mapping approach which is described above, DNAs from uninfected Vero and L tk^- Cl1D cells, as well as calf thymus DNA were processed through the preselection steps described above and hybridized to blots containing restriction enzyme fragments of viral DNA. Autoradiograms of these blots were devoid of identifiable bands (data not shown).

In an attempt to determine the sensitivity of this approach as regards unintegrated viral DNA sequences, either 3×10^{-2} or 3×10^{-1} μg of purified viral DNA (representing 1.0 and 10 copies per cell, respectively) were added to lysates of 2×10^8 Cl1D tk^- cells, and the resultant mixtures were sequentially processed as described above. The band patterns of these reconstruction hybridizations were compared to those of control hybridizations in which replicate nitrocellulose blots were hybridized to purified, in vitro-labeled HSV DNA. The results of these hybridizations are shown in Fig. 4 and 5 and revealed the following. (i) As seen in Fig. 4, the mapping approach is sufficiently sensitive to detect an unintegrated piece of HSV DNA of size 10^6 (e.g., *KpnI* fragment S, 1.1×10^6 in molecular weight), present in an average abundance of one copy per cell. (ii) In Fig. 5, we compared the relative molarities of the corresponding bands from the reconstruction and control hybridizations. As seen in that figure, there was no significant difference between the molarities of the control and reconstruction bands, indicating that the preselection steps did not result in the loss of specific subsets of viral DNA sequences. (iii) To determine whether the mapping approach described above would yield information concerning the number of copies per cell of the viral DNA sequences present in a given transformed cell line, we compared the absolute intensities of corresponding bands from autoradiograms of the 1 and 10 copy per cell reconstruction experiments. These ratios ranged from 7.9 to 21.9 (Table 2). Thus, the technique is quantitative within limits of approximately twofold error.

Because the buoyant density of cell-associated HSV DNA sequences depends upon whether these sequences are integrated into the host genome, it was also necessary to consider the sensitivity of the mapping approach for integrated transformed-cell associated HSV DNA sequences. Whether a specific sequence of integrated viral DNA will be recovered from the

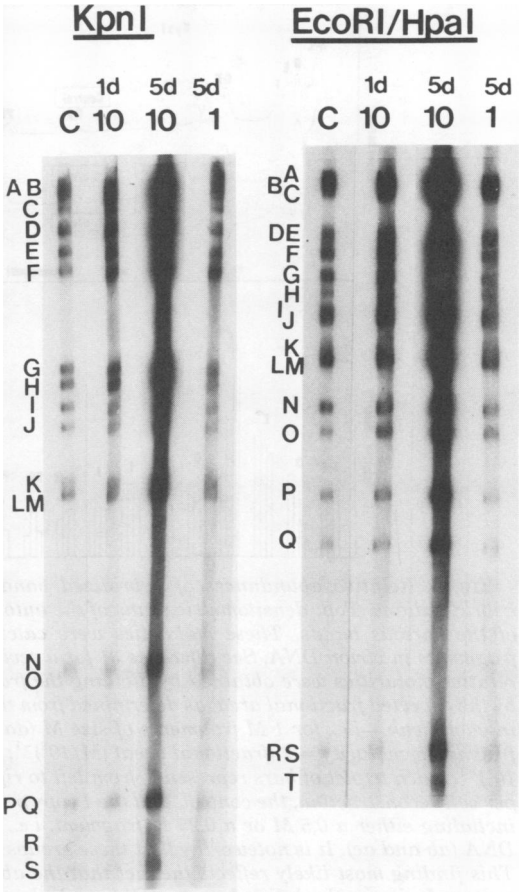

FIG. 4. *Hybridization reconstruction experiment. Either 3×10^{-2} or 3×10^{-1} μg of purified HSV-2 DNA (representing 1 and 10 copies per cell, respectively), were mixed with a lysate of 2×10^8 Cl1D tk^- cells. The resultant mixtures were preselected to enrich for viral DNA sequences and hybridized to nitrocellulose blots containing the KpnI or EcoRI/HpaI restriction enzyme fragments of HSV-2(G) DNA as described in the text. C = control: replicate blots which were hybridized to purified in vitro-labeled HSV-2 DNA, 10 = in vitro-labeled DNA from the 10 copy per cell reconstruction mixture, 1 = in vitro-labeled DNA from the 1 copy per cell reconstruction mixture, 1d = 1 day of autoradiographic exposure, and 5d = 5 days of autoradiographic exposure.*

CsCl density gradient purification step (i.e., will be included in pieces of sheared transformed-cell DNA with buoyant densities greater than or equal to 1.710 g/cm^3) will depend upon the exact size and density of the integrated viral DNA sequence, as well as upon the density of the surrounding cell DNA sequences. All of these parameters will, most probably, vary from cell line to cell line, and it is therefore impossible to

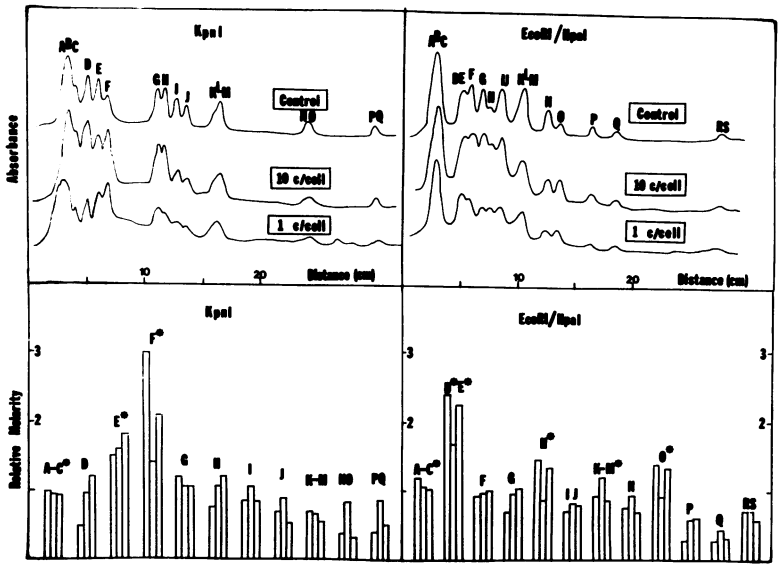

FIG. 5. *Relative abundances of hybridized bands from autoradiograms of control and reconstruction hybridizations. Top: densitometric scans of the autoradiograms shown in Fig. 4. Bottom: relative molarities of the various bands. These molarities were calculated relative to the molarities of the corresponding fragments in virion DNA. See reference 31 for a review of the relevant structural features of HSV DNA. The relative molarities were obtained by dividing the fractional area under each peak of the densitometric scan by the expected fractional area, as determined from the sizes and known molarities of the fragments contained in each peak,—i.e., for 1 M fragments of size M (daltons), molarity = (fractional area) $(M/10^8)^{-1}$; for 0.5 M fragments, molarity = 2 (fractional area) $(M/10^8)^{-1}$; for 0.25 M fragments, molarity = 4 (fractional area) $(M/10^8)^{-1}$. Each triplet of bars represents, from left to right, the relative molarities of the bands from the 10 copy per cell reconstruction, the control, and the 1 copy per cell reconstruction experiments. *, A group of fragments including either a 0.5 M or a 0.25 M fragment, i.e., containing sequences from the inverted repeats of HSV DNA (ab and ac). It is noteworthy that these groups of fragments exhibit relative molarities greater than 1.0. This finding most likely reflects the fact that the ab and ac repeated sequences are present in greater than unit molarity in the labeled probe DNA and, therefore, confirms the hypothesis that the hybridization is sensitive to the amounts of labeled probe DNA present in the hybridization solution.*

experimentally or theoretically determine the absolute sensitivity of the mapping approach for all cases of integrated viral DNA sequences. However, in the theoretical treatment presented above, we have calculated the fractional recovery (from the CsCl density gradient) of the viral DNA sequences contained in pieces of viral DNA of average buoyant density, which are integrated into cellular DNA of average buoyant density. These calculations predict that, by using our experimental conditions, any HSV DNA sequence which is present in a piece of integrated viral DNA of molecular weight greater than 3×10^6 will be recovered from the CsCl gradient preselection step with a minimum efficiency of 50%.

Mapping of the HSV-2 DNA sequences present in HSV-2tk$^+$ 33A$^+$ cells. Cell line 33A$^+$ was produced by infection of tk$^-$ mouse cells with UV light-irradiated HSV-2 (333). DNA extracted from passage 50 of this cell line was preselected and hybridized, as described above, to blots containing either the *Kpn*I or *Eco*RI/*Hpa*I restriction enzyme fragments of HSV-2 (G) DNA. The results of these hybridizations (Fig. 6) can be summarized as follows. (i) The maps generated from the two different restriction enzyme cleavages used in these experiments were in agreement and indicated that this cell line contains a contiguous set of HSV-2 (G) DNA sequences located between map coordinates 0.14 and 0.57. (ii) A comparison of the intensities of the bands produced by 33A$^+$ DNA with those produced by the 1 copy per cell reconstruction mixture, which was hybridized to replicate nitrocellulose blots, revealed that 33A$^+$ cells contain between 1 and 5 copies per cell of these HSV-2 DNA sequences (Fig. 6). (iii) To assess the reproducibility of the mapping approach, two additional preparations of 33A$^+$ DNA (passages 60–70) were separately preselected and hybridized to different sets of filters and blots containing HSV-2 DNA. The three preparations of transformed-cell DNA yielded identical hybridization patterns (Fig. 6).

Mapping of the viral DNA sequences present in three additional HSV-2tk$^+$ transformants. In an attempt to further delineate

TABLE 2. *Comparison of the intensities of the bands from the 1 and 10 copy per-cell reconstruction experiments*

Restriction endonuclease	Fragment	Intensity 10/intensity 1[a]
*Kpn*I	A	8.3
	B	
	C	
	D	16.44
	E	10.08
	F	8.43
	G	12.3
	H	
	I	8.27
	J	8.86
	K	8.03
	L	
	M	
	N	7.89
	O	
	P	12.2
*Eco*RI/*Hpa*I	A	7.99
	B	
	C	
	D	9.03
	E	
	F	9.94
	G	13.91
	H	8.54
	I	10.55
	J	
	K	9.27
	L	
	M	
	N	8.84
	O	9.45
	P	20.74
	Q	21.96
	R	10.0
	S	

[a] Ratios of the intensities of the corresponding bands (as determined by densitometric scanning as described in the text) of the 10 and 1 copy per cell reconstruction experiments shown in Fig. 1. The 5-day autoradiographic exposures were used for the densitometric scanning.

the map position of the tk gene of various strains of HSV-2, we mapped the viral DNA sequences which are present in late passages of three additional HSV-2tk+-transformed cell lines: 39A+ cells, produced by exposure of tk- cells to UV light-irradiated HSV-2 (333) and 59D+ and Silow cells, produced by infection of tk- cells with UV light-irradiated HSV-2 (324) and HSV-2 (Silow), respectively. The results of these mapping experiments (Fig. 7) revealed the following: 39A+ cells contained a contiguous set of viral DNA sequences located between map coordinates 0.14 and 0.42. Silow cells contained a contiguous stretch of viral DNA sequences mapping between coordinates 0.21 and 0.32. Finally, 59D+ cells contained a contiguous set of viral sequences spanning coordinates 0.28 to 0.42.

In addition to the viral DNA sequences described above, cell lines 39A+, 59D+, and Silow all contained two sets of viral DNA sequences which were present in significantly lower abundances (less than 0.1 copy per cell). These low-abundance viral DNA sequences have been tentatively mapped in two separate regions of the viral genome located between map coordinates 0.06 to 0.20 and 0.82 to 0.93.

A comparison of the map positions of the viral DNA sequences present in the four HSV-2tk+ transformants (Fig. 6 and 7) reveals that the only region of the HSV-2 genome which is common to all four cell lines is located between map coordinates 0.28 and 0.32. Thus, this region of the HSV-2 genome must contain the HSV-2 tk gene.

Mapping of the HSV-1 DNA sequences present in two HSV-1tk+-transformed cell lines. Cell lines 5A and 8N were produced by transfection of L tk- Cl1D mouse cells with HSV-1 (1023) DNA which was randomly sheared to fragments of molecular weight 20×10^6 to 30×10^6. DNAs from passage 15 of these cell lines were preselected and hybridized, as described above, to nitrocellulose blots containing various restriction enzyme fragments of HSV-1 DNA. Autoradiograms of the hybridized blots from these experiments (Fig. 8 and 9) revealed that (i) cell line 5A contains a contiguous set of HSV-1 DNA sequences mapping between coordinates 0.27 and 0.41, and (ii) cell line 8N contained four sets of noncontiguous *L* region viral DNA sequences mapping between coordinates 0.11 to 0.17, 0.29 to 0.32, 0.34 to 0.40, and 0.52 to 0.56. In addition, as seen in Fig. 9, 8N DNA hybridized to the *Bgl*II/*Hin*dIII fragment Q of HSV-1 (Justin) DNA, and to the *Bgl*II/*Hin*dIII fragment R, but not to the *Bgl*II/*Hin*dIII fragment P, of HSV-1 (F) DNA. Moreover, it hybridized to the 0.5 M fragments from the right side of the S region of HSV-1 DNA more efficiently than to those from the left side of the S region. Therefore, we have concluded that this cell contains a contiguous set of S region DNA sequences, composed of the U_s se-

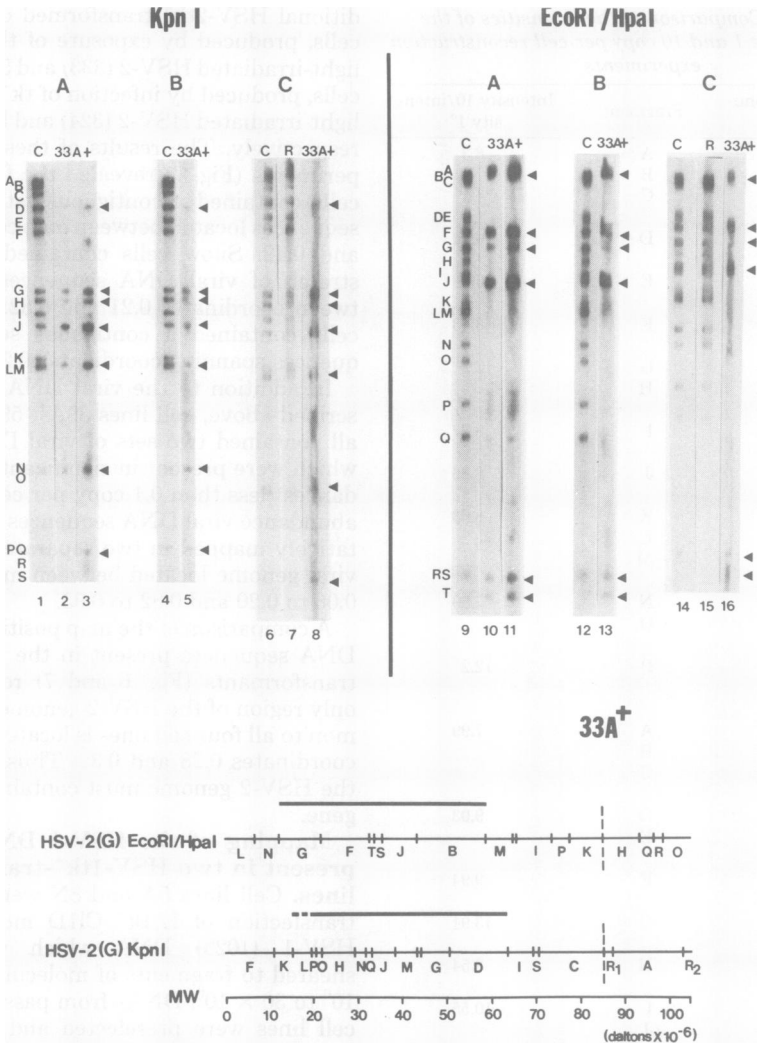

FIG. 6. *Mapping of the HSV-2 DNA sequences present in the HSV-2tk$^+$ cell line 33A$^+$. Top: control DNA (purified in vitro-labeled HSV-2 [G] DNA) (C) or in vitro-labeled 33A$^+$ DNA (33A$^+$) which was processed through the preselection steps was hybridized to nitrocellulose strips containing either the KpnI, or the EcoRI/HpaI restriction enzyme fragments of HSV-2(G) DNA. A, B, and C, Autoradiograms from three separate hybridization experiments, each done with a separate preparation of 33A$^+$ DNA (passages 50–70 of the cell line) and a separate set of nitrocellulose blots. In experiment C a 1 copy per cell reconstruction mixture (see legend to Fig. 4) was hybridized to a replicate nitrocellulose strip (R). In experiment A, autoradiography was performed for either 5 days (panels 2 and 10) or 3 weeks (panels 3 and 11). Bottom: schematic representation of the regions of homology between 33A$^+$ DNA and the KpnI, or EcoRI/HpaI restriction enzyme fragments of HSV-2 (G) DNA. The restriction enzyme maps were taken from G. S. Hayward, T. G. Buchman, and B. Roizman (unpublished data). The dotted line represents a region of uncertain homology, due to the comigration of the KpnI fragments L and M.*

quences mapping between coordinates 0.91 and 0.96 and a portion of the ac sequences.

DISCUSSION

In the studies reported in this paper, we have described a novel mapping approach which has allowed the simultaneous detection and identification of the HSV DNA sequences which are present in six HSVtk$^+$-transformed cell lines. Whereas this method requires relatively small amounts of transformed-cell DNA, it possesses sufficient sensitivity to detect a small piece of HSV DNA which is present in an average abundance of 1 copy per cell. Thus, this procedure could be applied in studies of cells transformed by other viruses containing genomes which dis-

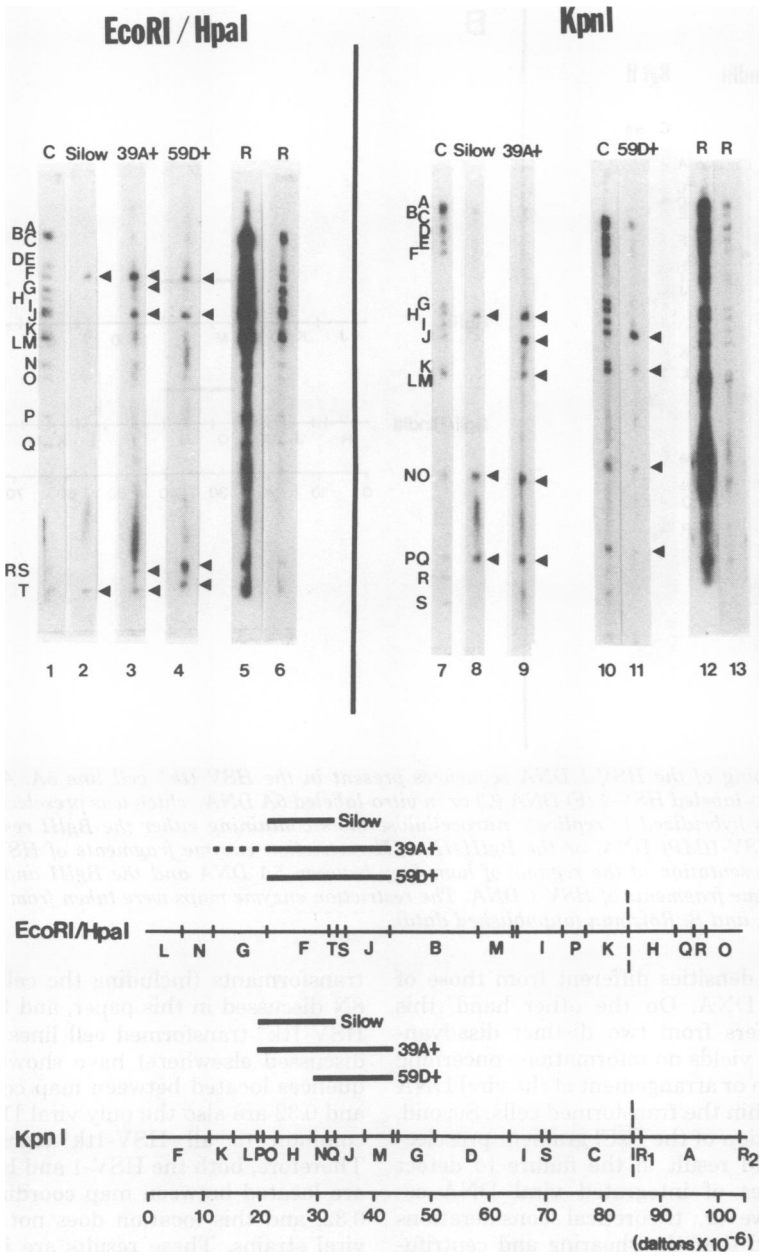

FIG. 7. *Mapping of the HSV-2 DNA sequences present in the HSV-2tk+ cell lines Silow, 39A+, and 59D+. Top: control [purified in vitro-labeled HSV-2(G) DNA] (panels 1, 5, and 8) or in vitro-labeled Silow (panels 2 and 6), 39A+ (panels 3 and 7), and 59D+ (panels 4 and 9) transformed-cell DNAs, which were preselected to enrich for viral DNA sequences, were hybridized to nitrocellulose blots containing either the KpnI or EcoRI/HpaI restriction enzyme fragments of HSV-2(G) DNA. Autoradiography was carried out for 4 days. Bottom: schematic representation of the regions of homology between Silow, 39A+, and 59D+ DNAs and the KpnI or EcoRI/HpaI restriction enzyme fragments of HSV-2 (G) DNA. The restriction enzyme maps were taken from G. S. Hayward, T. G. Buchman, and B. Roizman (unpublished data). Dotted lines represent a region of uncertain homology, due to comigration of the KpnI fragments L and M. It should be noted that as described in the text all three HSVtk+ cell DNAs showed low intensity hybridization to fragments located between map coordinates 0.06 to 0.20 and 0.82 to 0.93. Because of their extremely low abundances, these regions are not displayed in the bottom part of the figure.*

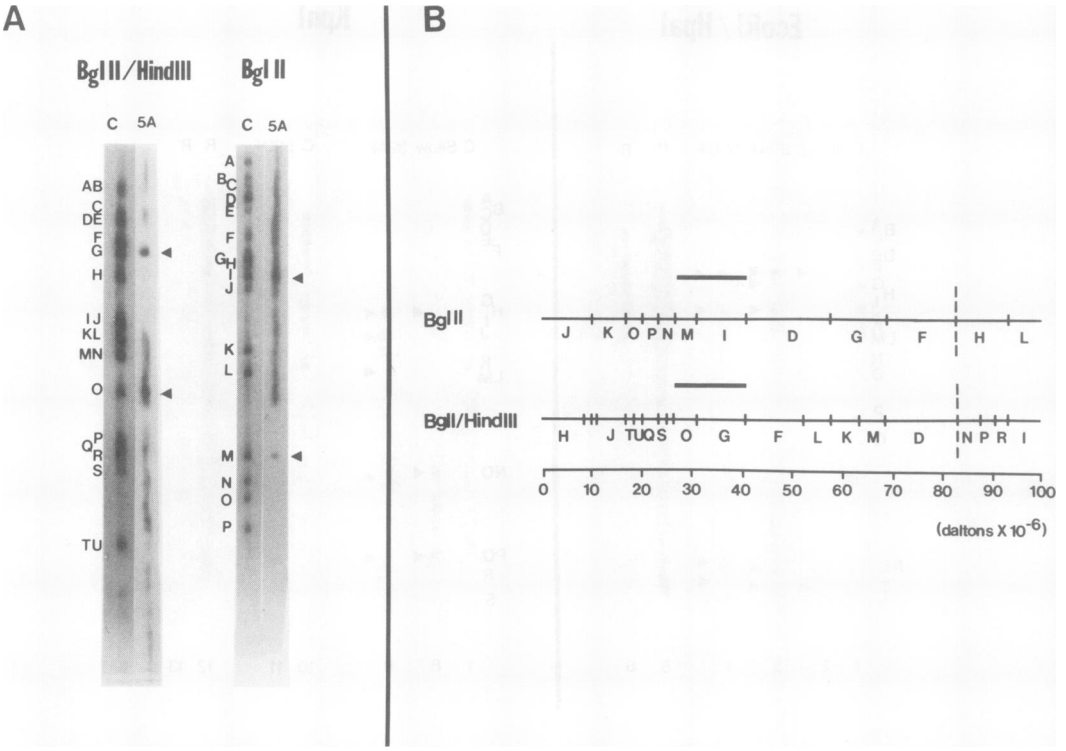

FIG. 8. *Mapping of the HSV-1 DNA sequences present in the HSV-1tk$^+$ cell line 5A. A, Control DNA (purified in vitro-labeled HSV-1 (F) DNA (C) or in vitro-labeled 5A DNA, which was preselected as described in the text, was hybridized to replicate nitrocellulose blots containing either the BglII restriction enzyme fragments of HSV-1(MP) DNA, or the BglII/HindIII restriction enzyme fragments of HSV-1(F) DNA. B, Schematic representation of the regions of homology between 5A DNA and the BglII and BglII/HindIII restriction enzyme fragments of HSV-1 DNA. The restriction enzyme maps were taken from G. S. Hayward, T. G. Buchman, and B. Roizman (unpublished data).*

play buoyant densities different from those of host cellular DNA. On the other hand, this approach suffers from two distinct disadvantages. First, it yields no information concerning the integration or arrangement of the viral DNA sequences within the transformed cells. Second, the incorporation of the CsCl gradient preselection step could result in the failure to detect small stretches of integrated viral DNA sequences. However, theoretical considerations have indicated that the shearing and centrifugation conditions used in the studies reported in this paper would have allowed the detection and mapping of pieces of integrated viral DNA with molecular weights greater than 3×10^6 (i.e., 3% of the HSV genome).

Mapping of the HSV-2 tk gene. The maps shown in Fig. 10 indicate that the sequences located between map coordinates 0.28 and 0.32 are the only viral DNA sequences which are common to all of the HSV-2tk$^+$-transformed cells. Similar mapping studies of the HSV DNA sequences which are present in six HSV-1tk$^+$ transformants (including the cell lines 5A and 8N discussed in this paper, and four additional HSV-1tk$^+$-transformed cell lines which will be discussed elsewhere) have shown that the sequences located between map coordinates 0.28 and 0.32 are also the only viral DNA sequences common to all HSV-1tk$^+$-transformed cells. Therefore, both the HSV-1 and HSV-2 tk genes are located between map coordinates 0.28 and 0.32, and this location does not vary between viral strains. These results are in accord with the findings of Morse et al. (25). However, they are in disagreement with the results of Maitland and McDougall (21).

Non-tk gene viral DNA sequences present in HSVtk$^+$ cells. In addition to the viral DNA sequences located between map coordinates 0.28 and 0.32, all of the HSVtk$^+$-transformed cell lines which we have tested contain a substantial, but variable, amount of non-tk gene viral DNA. Several points are noteworthy concerning the possible expression and function(s) of these viral DNA sequences. First, if

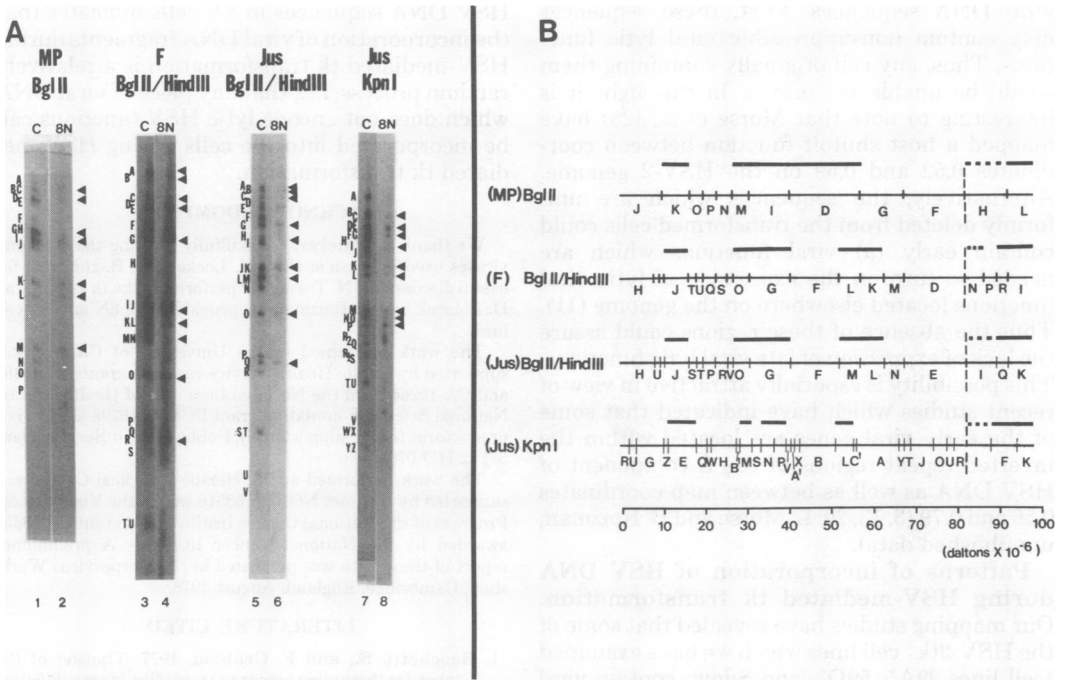

FIG. 9. *Mapping of the HSV-1 DNA sequences present in the HSV-1tk+ cell line 8N. A, Control DNA (purified in vitro-labeled HSV-1 DNA) or in vitro-labeled 8N DNA, which was preselected to enrich for viral DNA sequences, was hybridized to replicate nitrocellulose blots containing various restriction enzyme fragments of DNA from HSV-1 strains MP, F, and Justin (Jus). KpnI fragments R_1 and R_2 are both derived from the ends of the L region and reflect the heterogeneity of the viral DNA sequences in this region of the HSV-1 genome (35; H. Locker and N. Frenkel, manuscript in preparation). B, Schematic representation of the regions of homology between 8N DNA and the BglII, BglII/HindIII, and KpnI restriction enzyme fragments of HSV-1 DNA. The BglII and BglII/HindIII restriction enzyme maps were taken from G. S. Hayward, T. G. Buchman, and B. Roizman (unpublished data). The KpnI restriction enzyme map was taken from H. Locker and N. Frenkel (manuscript in preparation). Dotted lines represent regions of uncertain homology, which are presumably due to the presence of the inverted repeat ac sequences in 8N DNA (see text).*

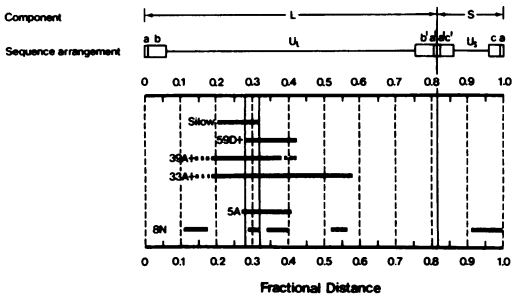

FIG. 10. *Summary of the mapping data of the HSV DNA sequences present in the six HSVtk+-transformed cell lines. The dotted lines represent a region of uncertain homology (see legend to Fig. 6).*

these sequences encode any of the known lytic functions of HSV, the expression of these lytic functions must be suppressed in the transformed cells. Second, it has been previously suggested that some of these non-tk gene viral DNA sequences may exert a positive regulatory effect on viral tk gene expression in these cells and may, therefore, be positively selected for during propagation of the cells in HAT medium (17). This possibility currently seems unlikely in light of the fact that none of the HSVtk+ cell lines tested in this study contain the viral DNA sequences which are known to encode the early (α) viral genes.

The results shown in Fig. 10, when taken together with the mapping data of four additional HSV-1tk+ transformants to be presented elsewhere, have also revealed that there are two regions of the viral genome which appear to be uniformly absent from most, if not all, HSVtk+ transformants. These regions are located between map coordinates 0 to 0.06, and 0.57 to 0.82. One can envision at least two alternative explanations for the uniform absence of these

viral DNA sequences. First, these sequences may contain nonsuppressible viral lytic functions. Thus, any cell originally containing them would be unable to survive. In this light it is interesting to note that Morse et al. (25) have mapped a host shutoff function between coordinates 0.52 and 0.59 on the HSV-2 genome. Alternatively, the sequences which are uniformly deleted from the transformed cells could contain early (α) viral functions which are needed to turn on the expression of lytic viral functions located elsewhere on the genome (11). Thus the absence of these regions could assure the lack of expression of late viral lytic functions. This possibility is especially attractive in view of recent studies which have indicated that some of the early viral genes are located within the inverted repeat regions of the *L* component of HSV DNA as well as between map coordinates 0.56 and 0.79 (3, 13, 29; L. Morse and B. Roizman, unpublished data).

Patterns of incorporation of HSV DNA during HSV-mediated tk transformation. Our mapping studies have revealed that some of the HSV-2tk$^+$ cell lines which we have examined (cell lines 39A$^+$, 59D$^+$, and Silow) contain viral DNA sequences which were present in very low abundance. One can envision at least two underlying mechanisms which could generate these observed abundance patterns. First, it is possible that each of these cell lines represents the product of several independent transformation events (of several different cells), all of which resulted in the incorporation of the tk gene, but each of which involved the incorporation of a different set of contiguous non-tk gene viral DNA sequences. This is possible given that the HSV-2tk$^+$ cell lines used in these studies were not extensively cloned. Alternatively, it is possible that these cell lines may have been the product of a single transformation event, but that following the initial cloning in HAT medium there was a period of loss of non-tk gene viral DNA sequences from selected cells within the originally uniform population. These possibilities are currently being differentiated by mapping studies of the DNA from subclones of late passages of these cell lines.

Finally, it should also be noted that most, if not all, of the UV light-irradiated HSV-produced cell lines appear to contain a single contiguous sequence of HSV DNA, whereas 8N cells which were produced by transfection of tk$^-$ cells with sheared HSV DNA clearly contain a large number of noncontiguous HSV DNA sequences. This difference might reflect the fact that DNA transfections result in the incorporation of multiple DNA fragments into the same cell. Moreover, the finding of the multiple noncontiguous sets of HSV DNA sequences in 8N cells indicates that the incorporation of viral DNA fragments during HSV-mediated tk transformation is a relatively random process, i.e., that any piece of viral DNA which does not encode lytic HSV functions can be incorporated into the cells during HSV-mediated tk transformation.

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LITERATURE CITED

1. **Bacchetti, S., and F. Graham.** 1977. Transfer of the gene for thymidine kinase to thymidine kinase-deficient human cells by purified herpes simplex viral DNA. Proc. Natl. Acad. Sci. U.S.A. **74**:1590–1594.
2. **Baker, R. F.** 1977. Binding of DNA to cellulose nitrate filters under denaturing conditions. Analyt. Biochem. **78**:569–571.
3. **Clements, J. B., R. J. Watson, and N. M. Wilkie.** 1977. Temporal regulation of herpes simplex virus type 1 transcription: location of transcripts on the viral genome. Cell **12**:275–285.
4. **Davidson, R., S. Adelstein, and M. Oxman.** 1973. Herpes simplex virus as a source of thymidine kinase for thymidine kinase deficient mouse cells: suppression and reactivation of the viral enzyme. Proc. Natl. Acad. Sci. U.S.A. **70**:1912–1916.
5. **Denhardt, D. T.** 1966. A membrane filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Comm. **23**:641–646.
6. **Dubbs, D. R., and S. Kit.** 1964. Mutant strains of herpes simplex deficient in thymidine kinase-inducing activity. Virology **22**:493–502.
7. **Garfinkle, B., and B. McAuslan.** 1974. Regulation of herpes simplex virus induced thymidine kinase. Biochem. Biophys. Res. Commun. **58**:822–829.
8. **Graham, F. L., A. J. Van der Eb, and H. L. Heijneker.** 1974. Size and location of the transforming region in human adenovirus type 5 DNA. Nature **251**:687–691.
9. **Groneberg, J., Y. Chardonnet, and W. Doerfler.** 1977. Integrated viral sequences in adenovirus type 12 transformed hamster cells. Cell **10**:101–111.
10. **Hayward, G.** 1974. Unique double-stranded fragments of bacteriophage T5 DNA resulting from preferential shear induced breakage of nicks. Proc. Natl. Acad. Sci. U.S.A. **39**:679–701.
11. **Honess, R. W., and B. Roizman.** 1975. Regulation of herpesvirus macromolecular synthesis. Sequential transition of polypeptide synthesis requires frunctional viral polypeptides. Proc. Natl. Acad. Sci. U.S.A. **72**:1276–1295.
12. **Hotta, Y., and A. Bassel.** 1965. Molecular size and circularity of DNA in cells of mammals and higher plants. Proc. Natl. Acad. Sci. U.S.A. **53**:356–362.

13. **Jones, P. C., G. S. Hayward, and B. Roizman.** 1977. Anatomy of herpes simplex virus DNA. VII. αRNA is homologous to non-contiguous sites in both L and S components of viral DNA. J. Virol. **21**:268–276.
14. **Kieff, E. D., S. Bachenheimer, and B. Roizman.** 1971. Size, composition and structure of the DNA of subtypes 1 and 2 herpes simplex virus. J. Virol. **8**:125–132.
15. **Kit, S., and D. R. Dubbs.** 1963. Acquisition of thymidine kinase activity by herpes simplex infected mouse fibroblast cells. Biochem. Biophys. Res. Commun. **11**:55–59.
16. **Klemperer, H. G., G. R. Haynes, W. I. H. Shedden, and D. H. Watson.** 1967. A virus specific thymidine kinase in BHK21 cells infected with herpes simplex virus. Virology **31**:120–128.
17. **Leiden, J., R. Buttyan, and P. G. Spear.** 1976. Herpes simplex virus gene expression in transformed cells. I. Regulation of viral thymidine kinase gene in transformed L cells by products of superinfecting virus. J. Virol. **20**:413–424.
18. **Leung, W. C.** 1978. Evidence for a herpes simplex virus-specific factor controlling the transcription of deoxypyrimidine kinase. J. Virol. **27**:269–274.
19. **Lin, S.-S. and W. Munyon.** 1974. Expression of the viral thymidine kinase gene in herpes simplex virus transformed L cells. J. Virol. **14**:1199–1208.
20. **Littlefield, J.** 1964. Selection of hybrids from matings of fibroblasts *in vitro* and their presumed recombinants. Science **145**:709–710.
21. **Maitland, N. J., and J. K. McDougall.** 1977. Biochemical transformation of mouse cells by fragments of herpes simplex virus DNA. Cell **11**:233–241.
22. **Maniatis, T., A. Jeffrey, and D. G. Kleid.** 1975. Nucleotide sequences of the rightward operator of phage λ. Proc. Natl. Acad. Sci. U.S.A. **72**:1184–1188.
23. **Minson, A. C., P. Wildy, A. Buchan, and G. Darby.** 1978. Introduction of the herpes simplex virus thymidine kinase gene into mouse cells using virus DNA or transformed cell DNA. Cell **13**:581–587.
24. **Morse, L. S., T. G. Buchman, B. Roizman, and P. A. Schaffer.** 1977. Anatomy of herpes simplex virus DNA. IX. Apparent exclusion of some parental DNA arrangements in the generation of intertypic (HSV-1 × HSV-2) recombinants. J. Virol. **24**:231–248.
25. **Morse, L. S., L. Pereira, B. Roizman, and P. A. Schaffer.** 1978. Anatomy of HSV DNA. X. Mapping of viral genes by analysis of polypeptides and functions specified by HSV-1 × HSV-2 recombinants. J. Virol. **26**:389–410.
26. **Munyon, W., R. Buchsbaum, E. Paoletti, J. Mann, E. Kraiselburd, and D. Davis.** 1972. Electrophoresis of thymidine kinase activity synthesized by cells transformed by herpes simplex virus. Virology **49**:683–689.
27. **Munyon, W., E. Kraiselburd, D. Davis, and J. Mann.** 1971. Transfer of thymidine kinase to thymidine kinase-less L cells by infection with ultraviolet-irradiated herpes simplex virus. J. Virol. **7**:813–820.
28. **Ogino, T., R. Shiman, and F. Rapp.** 1973. Deoxythymidine kinase from rabbit kidney cells infected with herpes simplex virus types 1 and 2. Intervirology **1**:80–95.
29. **Preston, V. G., A. J. Davison, H. S. Marsden, M. C. Timbury, J. H. Subak-Sharpe, and N. M. Wilkie.** 1978. Recombinants between herpes simplex virus types 1 and 2: analyses of genome structures and expression of immediate early polypeptides. J. Virol. **28**:499–517.
30. **Rapp, F., and N. Turner.** 1978. Biochemical transformation of mouse cells by herpes simplex virus types 1 and 2: comparison of different methods for inactivation of viruses. Arch. Virol. **56**:77–87.
31. **Roizman, B.** 1979. The structure and isomerization of herpes simplex virus genomes. Cell **16**:481–494.
32. **Ruyechan, W. T., L. S. Morse, D. M. Knipe, and B. Roizman.** 1979. Molecular genetics of herpes simplex virus. II. Mapping of the major viral glycoproteins and of the genetic loci specifying the social behavior of infected cells. J. Virol. **29**:677–697.
33. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
34. **Thouless, M. and P. Wildy.** 1975. Deoxypyrimidine kinases of herpes simplex viruses type 1 and 2: comparison of serological and structural properties. J. Gen. Virol. **26**:159–170.
35. **Wagner, M. J., and W. C. Summers.** 1978. Structure of the joint S region and the termini of the DNA of herpes simplex virus type 1. J. Virol. **27**:374–387.
36. **Wetmur, J. G.** 1975. Acceleration of DNA renaturation rates. Biopolymers **14**:2517–2524.
37. **Wigler, M., S. Silverstein, L. Lee, A. Pellicer, Y. Cheng, and R. Axel.** 1977. Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. Cell **11**:223–232.